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# L-LEUCYL-L-LEUCINE METHYL ESTER TREATMENT OF CANINE MARROW AND PERIPHERAL BLOOD CELLS

## INHIBITION OF PROLIFERATIVE RESPONSES WITH MAINTENANCE OF THE CAPACITY FOR AUTOLOGOUS MARROW ENGRAFTMENT[1]

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**Incubation of canine marrow and peripheral blood mononuclear cells with L-leucyl-L-leucine methyl ester resulted in the inhibition of mitogen- and alloantigen-induced blastogenesis, the elimination of allosensitized CTL and NK activity, and prevented the development of CTL from pCTL. The effects of these incubations were similar to those described in mice and humans. Additionally, in vitro CFU-GM growth from treated canine marrow was reduced, but could be regained when the Leu-Leu-OMe–treated marrow was cocultured with either untreated autologous peripheral blood mononuclear cells or monocyte-enriched PBMC but not with untreated monocyte-depleted PBMC. Six of seven dogs conditioned with 920 cGy total-body irradiation engrafted successfully after receiving autologous marrow that was incubated with Leu-Leu-OMe prior to infusion. These cumulative results indicate that incubation with Leu-Leu-OMe is a feasible method to deplete canine marrows of alloreactive and cytotoxic T cells prior to transplantation.**

The success of allogeneic marrow transplantation as treatment for malignant and nonmalignant hematopoietic diseases has been restricted by the serious complications of graft-versus-host disease (*1, 2*). Experiments in a variety of mammalian marrow transplant models have shown that removal of mature T cells from donor marrow permits engraftment without the development of GVHD (*3–6*). Based on these and similar observations, studies have been carried out to evaluate the effects of T cell depletion prior to allogeneic marrow transplantation in humans. Most studies have employed marrow treatments with anti-T cell monoclonal antibodies plus complement or with soybean agglutinin followed by E rosette formation and density gradient centrifugation (*7–9*). In general, removal of T cells has been associated with a marked decrease in both the incidence and severity of GVHD. However, the use of T cell–depleted marrow has also been associated with an increased incidence of marrow graft rejection (*10*).

Recently, Thiele and Lipsky have described a dipeptide methyl ester, L-leucyl-L-leucine-methyl ester (Leu-Leu-OMe)* that can eliminate natural killer cells (NK), monocytes (Mø), and precursors of alloantigen-specific cytotoxic T cells (pCTL) from mouse spleen cell suspensions and from both mouse and human peripheral blood. This treatment leaves intact B cells, helper T cells, and murine erythroid and hematopoietic stem cells (*11–14*). In a murine histoincompatible marrow transplant model (C57BL/6J→(C57BL/6×DBA/2)$F_1$), treatment of donor marrow and spleen cells with Leu-Leu-OMe resulted in successful donor marrow engraftment and the development of stable long-term hematopoietic chimerism without GVHD (*14–16*). The use of Leu-Leu-OMe to treat marrow may have advantages over currently used methods. The use of Leu-Leu-OMe is very simple, requiring but a single 15-min incubation. In addition, it appears that marrow incubation with Leu-Leu-OMe results in the elimination of the cells responsible for acute GVHD while at the same time preserving hematopoietic stem cells needed for engraftment and the cells required for immune reconstitution (*15, 16*).

We and others have used dogs as a large, outbred animal model for us in experimental marrow transplantation (*17, 18*). The present studies were undertaken to determine whether the incubation of canine marrow and peripheral blood cells with Leu-Leu-OMe would yield alterations of in vitro cellular immune function comparable to those described in human and murine cells and to investigate the effects of marrow incubation with Leu-Leu-OMe on early hematopoietic progenitors and stem cells assayed for both in vitro and in vivo function.

## MATERIALS AND METHODS

*Dogs.* Beagles, hounds, and mixed breed hounds, obtained from commercial vendors in Washington and Virginia or raised at the Fred Hutchinson Cancer Research Center (FHCRC), were dewormed and vaccinated against distemper, hepatitis, leptospirosis, and parvovirus before use in this study. All dogs were at least six months of age and were maintained at the FHCRC canine kennel facilities per guidelines stipulated by the National Academy of Sciences—National Research Council. The research protocol was approved by the Internal Animal Care and Use Committee at the Fred Hutchinson Cancer Research Center.

*Medium.* Waymouth's MB752/1 medium (FHCRC media preparation facility), supplemented with 0.1 mM nonessential amino acids and 100 U/ml penicillin and 100 μg/ml streptomycin (all from GIBCO),

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* Abbreviations: Leu-Leu-OMe, L-leucyl-L-leucine-methyl ester; Mø, monocytes; B-MLC, bulk MLC; NWNA, nylon-wool nonadherent; CFU-C, colony forming unit in culture; PEDS, postendotoxin dog serum; CTAC, canine thyroid adenocarcinoma cell line.

was used for the dilution of heparinized whole blood and marrow for the cell separation procedures. Waymouth's medium supplemented as above with the addition of 10% to 20% heat-inactivated (56°C) normal pooled dog serum (M-NPS/10–20%) was used for the mixed leukocyte culture microassays, bulk MLC (B-MLC), cell-mediated lympholysis, and NK assays.

*Cell preparation.* Peripheral blood mononuclear cells were obtained by the centrifugation of heparinized venous whole blood (diluted 1:2 with medium) over Ficoll-Hypaque density gradients (Sp. density 1.074) as previously described (*19*). Bone marrow cells (BMC) for in vitro assays were obtained by syringe aspiration from the humeral head of an anesthetized dog. The marrow was diluted 1:2 with medium and overlayed onto Ficoll-Hypaque density gradients for centrifugation (1000 × g), following which the interface cells were washed once with hemolytic buffer and twice with medium. The PBMC and marrow cells were resuspended into medium for cell counts and viability assessment using the trypan-blue exclusion technique.

Monocyte-enriched cells were obtained by treating PBMC with the anticanine murine monoclonal antibody Dly 6 (*20*) as follows: $300\times10^6$ PBMC were incubated for 30 min at room temperature in 30 ml of 1:100 diluted Dly 6 (ascites containing antibody), and then an equal volume of 1:2 diluted rabbit serum complement (Pel Freeze, Rogers, AR) was added for an additional 60 min. The cells were washed once with medium, resuspended in 30 ml of 1:3 diluted rabbit serum complement, and incubated again for 60 min. After washing twice in medium, these cell suspensions contained 61±8% (SEM) viable monocytes, 16±3% lymphocytes, and 23±9% granulocytes, primarily eosinophils.

Monocyte-depleted PBMC were obtained by first passing PBMC over nylon-wool columns as previously described (*21*), and then transferring $30\times10^6$ nylon-wool-nonadherent (NWNA) cells in 10 ml of medium, containing 5% fetal calf serum, into plastic petri dishes (Falcon No. 3003, Lincoln Park, NJ) for 2 hr incubation at 37°C, 7% $CO_2$. This depletion technique yielded approximately 96±1% lymphoid cells with greater than 90% viability and less than 3% monocytes as determined by morphologic assessment of Wright-stained cytospin preparations.

*Preparation of Leu-Leu-OMe.* The Leu-Leu-OMe was synthesized from L-leucyl-L-leucine (Sigma Chemical Co, St Louis, MO) as previously described (*11*). Qualitative assessment of Leu-Leu-OMe purity was obtained by thin-layer chromatography (TLC) (*22*). Briefly, 5 λ of $5\times10^{-3}$ M solutions of L-leucine methyl ester (Leu-OMe) (dissolved in absolute methanol), L-leucine (Leu), L-leucyl-L-leucine (Leu-Leu) (both dissolved with heat and stirring in absolute methanol containing 0.5 N HCl), and the synthesized Leu-Leu-OMe were applied to precoated TLC plates (250 μM, 10×20-cm, HPTLC Kieselgel 60 (Merck), Darmstadt, West Germany), and quickly dried under a stream of warm air. The plates were developed for 2.5 hr in an enclosed, equilibrated system containing the following mixture of reagent grade solvents: chloroform, absolute methanol and acetic acid at volume ratios of 19:0.6:12.5, respectively. The migrations of the four compounds were visualized by applying an aerosol spray of 0.2% ninhydrin in ethanol and then placing the plates in a 60°C oven for 30 min. $R_f$ values (the ratio of the distance the compound travels to the distance the solvent front travels) were calculated, in order to assess the resultant migrations, according to the following formula (*22*):

$$R_f = \frac{\text{distance traveled by compound}}{\text{distance traveled by solvent front}}$$

Leu-Leu-OMe was stored at −20°C in absolute methanol and, based on repeated TLC analysis, was stable for at least three months.

*Incubation of PBMC or marrow cells with Leu-Leu-OMe.* Equal volumes of PBMC or marrow cell suspensions and Leu-Leu-OMe at the indicated final concentrations were incubated for 15 min at room temperature. Cells for in vitro studies were washed twice and resuspended in medium. Marrow cells used for autologous infusion were incubated at cell concentrations of $20\times10^6$/ml in Leu-Leu-OMe solutions that contained 0.1 U/ml DNAase (Worthington Enzymes and Biochemicals, Freehold NJ). After incubation, these cells were washed, counted, and reinfused within 1–3 hr.

*Mixed leukocyte culture and mitogen assays.* MLCs were established, labeled with [$^3$H]thymidine, harvested, and prepared for liquid scintillation counting as previously described (*23*) with minor modifications. The $10^5$ Leu-Leu-OMe-treated or untreated responder and $10^5$ irradiated (2300 rads) untreated, stimulator PBMC were cocultured in a final volume of 200 μl M-NPS/20% per well. Mitogen stimulation was assessed by adding either 375 μg/ml PHA (DIFCO, Detroit MI), 200 μg/ml Con A, (Calbiochem, San Diego CA), or 200 μg/ml PWM (GIBCO, Grand island, NY) to $10^5$ treated or untreated responder cells in a final volume of 200 μl of M-NPS/20%. All cultures were established in triplicate in microtiter plates (Costar No. 3799, Cambridge MA) for 7 days at 37°C, 7% $CO_2$, in a humidified incubator.

*Bulk MLC (B-MLC) and cell-mediated lympholysis assays.* Bulk MLCs were established using either untreated or Leu-Leu-OMe–treated PBMC or marrow cells as responders, and untreated, irradiated PBMC as stimulators, to generate CTL for CML assays, as previously described (*19*), with modifications. CTL were derived from these cultures to form two CML assay groups: (1) responder PBMC or marrow cells treated with Leu-Leu-OMe or MeOH on day 0 prior to mixing with irradiated stimulator PBMC in B-MLC (day 0); and (2) Leu-Leu-OMe or MeOH treatment of 7-day B-MLC generated CTL (day 7). CTLs were mixed at a 50:1 effector:target ratio, with $^{51}$Cr-labeled $^{51}$Na $CrO_4$ (300–500 μCi/ml, NEN, Wilmington DE) Con A–stimulated PBMC targets. Cultures of PBMC to be used for targets in the CML assays were established on the same day of B-MLC, and stimulated with Con A on day 4 of culture. The 4-hr $^{51}$Cr release assay was performed as previously described (*19*). The mean spontaneous/maximum $^{51}$Cr release ratio was 16±1% (± SEM), while maximum release/total $^{51}$Cr incorporation was 90±2% for targets used in this series of CML assays.

Proliferation of treated or untreated responder PBMC or marrow cells in 7-day B-MLC was measured by distributing 200-μl aliquots from each B-MLC flask into triplicate microculture wells and labeling for 7 hr with [$^3$H]thymidine. Cell harvest and liquid scintillation counting were performed as described for MLC (*23*).

*Natural killer cell (NK) assays.* Leu-Leu-OMe or MeOH treated or untreated PBMC and marrow cells were assayed for NK activity against a $^{51}$Cr-labeled canine thyroid adenocarcinoma cell line (CTAC) in a 18-hr assay with the percentage of $^{51}$Cr release calculated as previously described (*24*).

*In vitro marrow cultures for CFU-GM growth.* Leu-Leu-OMe and MeOH treated and untreated marrow cells were tested for in vitro hematopoietic progenitor growth using an agar-based colony formation assay that utilizes postendotoxin dog serum (PEDS) as the source of colony-stimulating factor (*25*). Growth of granulocyte/macrophage colonies (CFU-GM) was assayed in cultures containing $10^5$ or $3\times10^5$ treated or untreated marrow cells only, and in cultures in which marrow cells were cocultured with either $10^5$ autologous PBMC, Mø-enriched or Mø-depleted PBMC, or Leu-Leu-OMe treated PBMC (incubated with 1000 μM Leu-Leu-OMe). Cultures were incubated in triplicate for 10 days in a 37°C, 7% $CO_2$ humidified incubator and GFU-GM colonies were enumerated as previously described (*25*).

*Autologous marrow transplantation.* Marrow for autologous transplantation was obtained by a vacuum pump aspiration procedure (*26*), processed over a Ficoll-Hypaque density gradient and incubated with Leu-Leu-OMe. All recipients were conditioned with 920 cGy total-body irradiation delivered as a single exposure from two opposing $^{60}$Co sources at 7.0 cGy/min (*26*). The recipients were infused with treated autologous marrow within 3 hr of TBI. Supportive care pre- and posttransplantation with antibiotics, i.v. fluids, and whole-blood transfusions, was given as previously described (*27*). In addition, recipients were given oral antibiotics (Neomycin sulfate and polymyxin B sulfate) daily for five days before TBI and posttransplant until the granulocyte count reached 500/mm$^3$.

## RESULTS

*Thin-layer chromatographic analysis of synthesized Leu-Leu-OMe.* Three batches of Leu-Leu-OMe were synthesized for use in the in vitro studies and autologous transplant experiments described. Consistent $R_f$ values were obtained for each batch of Leu-Leu-OMe and for the three control compounds tested. Within the Leu-Leu-OMe, there was a secondary spot that migrated with a $R_f$ value equal to that observed for the L-Leucyl-L-leucine. The consistency of the $R_f$ values indicated that constant yield and purity was achieved with minimal batch-to-batch variations.

*Viability of PBMC and marrow cells after incubation with Leu-Leu-OMe.* The viability of treated PBMC and marrow cells was assessed within 1 hr after incubation with Leu-Leu-OMe. There was no significant difference in viabilities observed in cells treated with either Leu-Leu-OMe (up to 1000 μM), 0.5% MeOH in PBS, or PBS only. No time-course studies were done to assess the viability of PBMC and marrow cells more than 1 hr after incubation with Leu-Leu-OMe.

*Elimination of alloantigen responsiveness and mitogen-induced lymphocyte blastogenesis by incubation of PBMC with Leu-Leu-OMe.* PBMC were treated at either $2\times10^6$ or $20\times10^6$/ml with Leu-Leu-OMe and tested in micro-MLC, and blastogenesis assays (Fig. 1). In all assays there was a Leu-Leu-OMe dose-dependent reduction in the proliferative response such that virtually no blastogenesis was observed after incubation with 1000 μM Leu-Leu-OMe.

Treatment of $20\times10^6$ responder PBMC or marrow cells before bulk MLC resulted in similar Leu-Leu-OMe dose-dependent reductions in proliferative response (data not shown). The marrow cells gave a lower baseline level of [$^3$H]thymidine incorporation and were more sensitive than the PBMC to treatment with Leu-Leu-OMe (data not shown). Treatment of PBMC marrow cells with 0.5% MeOH in PBS had no effect on the alloproliferative response in B-MLC (data not shown).

*Elimination of the generation of antigen-specific cytotoxic T lymphocytes by incubation of PBMC and marrow cells with Leu-Leu-OMe.* The effect of Leu-Leu-OMe on the generation of antigen-specific cytotoxic T cells was measured by incubating responder PBMC and marrow cells with Leu-Leu-OMe (day 0 treatment), and then testing these cells in a standard CML assay after 7 days of culture in B-MLC. Treatment with 1000 μM Leu-Leu-OMe eliminated the generation of cytolytic activity against $^{51}$Cr-labeled alloantigen-specific Con A-stimulated PBMC (Fig. 2). These results indicated that the generation of CTL from precursors is sensitive to incubation with Leu-Leu-OMe. The effect of Leu-Leu-OMe incubation on CTL already generated in 7-day bulk MLC (day 7 treatment) was also evaluated. This treatment eliminated antigen-specific cytolytic activity (Fig. 2). Treatment with 0.5% MeOH, either on day 0 or day 7, did not interfere with either the development of CTL or the specific cytolysis of targets assayed on day 7 (data not shown).

*Elimination of NK activity after incubation of PBMC and marrow cells with Leu-Leu-OMe.* Marked dimunition of NK activity, in a dose-dependent manner, was seen after incubation of PBMC or marrow cells with Leu-Leu-OMe, and NK activity was essentially eliminated after treatment with 1000 μM Leu-Leu-OMe (Fig. 3).

*In vitro CFU-GM colony growth reduction by Leu-Leu-OMe*

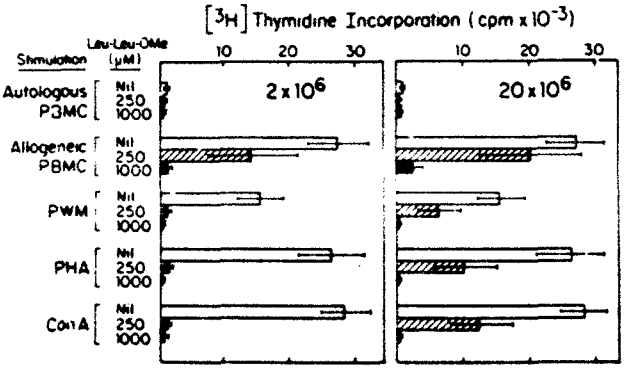


FIGURE 1. Effect of Leu-Leu-OMe on alloantigen- and mitogen-induced lymphocyte blastogenesis. Data for [$^3$H]thymidine incorporation are mean cpm±SEM from multiple assays in which PBMC were treated at concentration of $2\times10^6$ or $20\times10^6$ cells/ml.

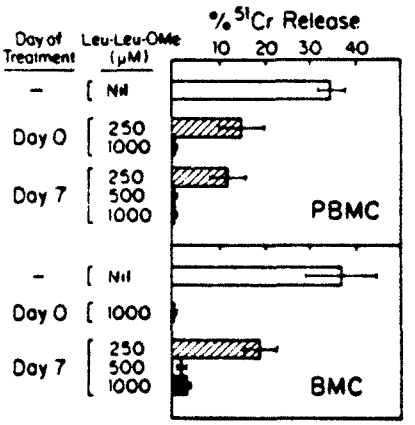


FIGURE 2. CML assays illustrating the effect of Leu-Leu-OMe treatment on p-CTL (day 0) or on 7-day B-MLC generated CTL (day 7), derived either from PBMC or BMC. The ordinate indicates mean percentage of $^{51}$Cr release ± SEM using specific alloantigen-sensitizing $^{51}$Cr-labeled Con A-stimulated PBMC as targets.

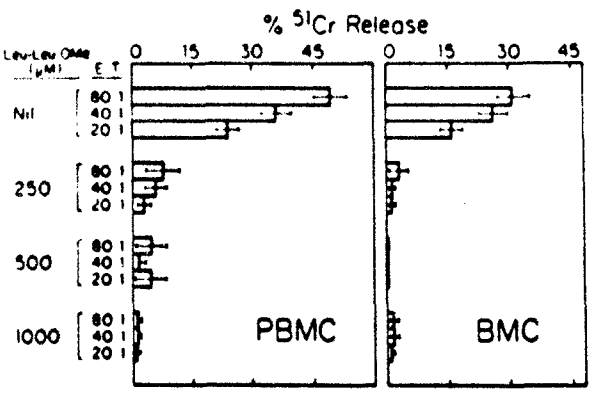


FIGURE 3. Leu-Leu-OMe treatment of PBMC and BMC eliminates NK cytolytic activity. The ordinate indicates mean percentage of $^{51}$Cr release ± SEM from CTAC at three different effector:target (E:T) ratios. For this series of assays, the mean spontaneous/maximum $^{51}$Cr release ratio was 23±1% (± SEM) and the maximum release/total $^{51}$Cr incorporation ratio was 66±7%.

*treatment of marrow cells.* There was a marked reduction in CFU-GM colony growth obtained from marrow cells incubated with varying concentrations of Leu-Leu-OMe (Table 1). Minimal CFU-GM growth was observed with $10^5$ Leu-Leu-OMe treated marrow cells per plate were cultured. There was some recovery of CFU-GM growth when the cell number was increased to $3\times10^5$ treated marrow cells per plate, but only to a level that was approximately 25% of that observed with $3\times10^5$ untreated marrow cells. Addition of either untreated, autologous PBMC or Mø-enriched autologous PBMC increased CFU-GM growth, but not to the levels observed with untreated marrow cells in similar cocultures. The addition of Mø-depleted or Leu-Leu-OMe treated autologous PBMC to treated marrow cells did not augment growth.

*The effect of incubating marrow cells with Leu-Leu-OMe on autologous marrow engraftment.* Six of the seven dogs given Leu-Leu-OMe treated autologous marrow engrafted and did so with kinetics similar to those seen in recipients of untreated autologous marrow (Table 2 and Fig. 4). Platelet counts returned to normal levels between 20 to 30 days posttransplant. Dog C521 did not survive past day 20 posttransplant. After Leu-Leu-OMe incubation, the marrow cells from this dog were clumped and had only 20% viability, resulting in the infusion of a very low marrow cell dose. The marrow from C580 was incubated with the same concentration of Leu-Leu-OMe as C521, but the treated marrow cells of C580 did not clump and this dog survived with rapid engraftment. Dog BB8701 had to be euthanized on day 19 posttransplant due to an accidental, severe, foot injury that failed to respond to treatment, but did show evidence of engraftment as indicated by a rise in WBC and the marrow cellularity at autopsy.

## DISCUSSION

Thiele and Lipsky have demonstrated that exposure of mouse spleen cells or human peripheral blood leukocytes to Leu-Leu-OMe depletes these populations of monocytes, NK cells, and cytotoxic lymphocytes at both the precursor and effector stages of differentiation, without apparently affecting other cell populations (*11–13*). They further showed that incubation of mixtures of murine marrow and spleen cells with Leu-Leu-OMe did not interfere with engraftment and could, in certain circumstances, prevent GVHD (*14–16*). In the studies presented here, we found that canine peripheral blood and marrow cells behave similarly following exposure to Leu-Leu-OMe with the elimination of functional monocytes, NK cells, and alloantigen-sensitized CTL, and inhibited the development of CTL from pCTL. Further, we found that incubation of marrow cells with Leu-Leu-OMe (even at very high doses) did not inhibit autologous engraftment in recipients conditioned with 920 cGy TBI. These studies also demonstrated that the treatment of canine

TABLE 1. In vitro CFU-GM colony growth from marrow cells incubated with varying concentrations of Leu-Leu-OMe

| Leu-Leu-OMe (μM)[c] | CFU-GM colonies obtained from CFU-C cultures[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $10^5$ BMC cocultures[b] | | | | | $3\times10^5$ BMC cocultures[b] | | | | |
| | No coculture | $10^5$ PBMC | $10^5$ Mø enriched | $10^5$ Mø depleted | $10^5$ Leu-Leu-OMe-treated PBMC | No coculture | $10^5$ PBMC | $10^5$ Mø enriched | $10^5$ Mø depleted | $10^5$ Leu-Leu-OMe-treated PBMC |
| Nil | 66±13 | 84±9 | 98±15 | 76±23 | 78±13 | 207±15 | 255±18 | 348±9 | 288±7 | 252±17 |
| 250 | 0.4±0.2 | 64±32 | | | | 55±31 | 178±63 | | | |
| 500 | 0 | 14±8 | | | | 3±2 | 51±24 | | | |
| 1000 | 1.2±0.5 | 10±3 | 29±11 | 2±1 | 2±0.6 | 8±3 | 42±3 | 74±29 | 0.3±0.3 | 3±1 |
| 2000 | 2±1 | 5±2 | | | | 2±0.8 | 13±5 | | | |
| 4000 | 0.7±0.6 | 11±5 | | | | 0.9±0.5 | 16±9 | | | |

[a] Represented are mean values ± SEM, obtained from multiple experiments in which triplicate CFU-GM cultures were established for each parameter tested.

[b] All cocultures employed autologous cells. The limited yield of Mø prevented coculture with all concentrations of Leu-Leu-OMe tested. There was no CFU-GM growth when untreated PBMC (between $10^4$ to $6\times10^4$/ml) were cultured without marrow cells.

[c] The concentration of marrow cells treated was constant at $20\times10^6$/ml.

TABLE 2. Recipients conditioned with 920 cGy TBI and receiving Leu-Leu-OMe treated autologous marrow[a]

| Dog ID | [Leu-Leu-OMe][b] (μM) | No. viable treated BMC infused ($10^8$/kg)[c] | Survival (days post-BMT) | Cause of death | Marrow cellularity |
|---|---|---|---|---|---|
| C563 | 1000 | 1.4 | 152 | Sodium pentothal[d] | Normocellularity (3 cell lines) |
| BB6701 | 1000 | 0.7 | 19 | Sodium pentothal[d] | 75% of Normocellularity (3 cell lines) |
| C822[e] | 1000 | 1.41 | >124 | Still living | Normocellularity (3 cell lines) |
| C521 | 2000 | 0.062 | 20 | Pneumonia; sepsis | Focal hematopoiesis |
| C580 | 2000 | 1.67 | 173 | Sodium pentothal[d] | Normocellularity (3 cell lines) |
| B960 | 4000 | 1.3 | 114 | Sodium pentothal[d] | Normocellularity (3 cell lines) |
| C862 | 4000 | 1.5 | 112 | Sodium pentothal[d] | Normocellularity (3 cell lines) |

[a] Autologous marrow aspiration, BMC treatment, and infusion on same day as 920 cGy TBI to recipient (BMT: bone marrow transplant).

[b] $20\times10^6$ BMC/ml treated with Leu-Leu-OMe.

[c] Viability of bone marrow cells infused determined by trypan-blue stain exclusion technique.

[d] Sodium pentothal injection for euthanasia at end of study.

[e] The Leu-Leu-OMe-treated autologous marrow was frozen and stored at −80°C for one week prior to reinfusion. The procedure for marrow cryopreservation was as described (*26*).

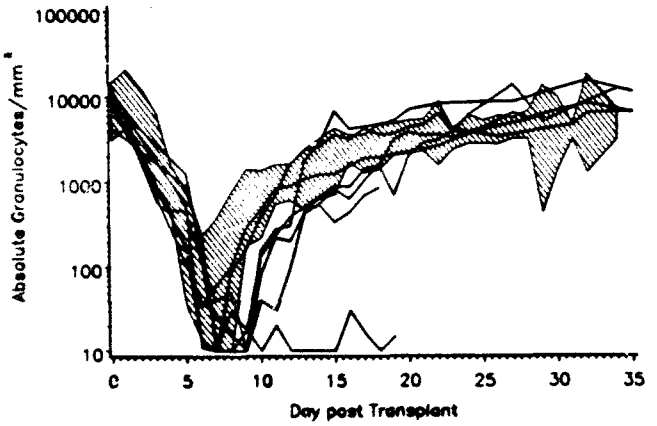


FIGURE 4. Hematologic recovery in recipients conditioned with 920 cGy TBI and infused with Leu-Leu-OMe-treated autologous marrow. The ordinate indicates the absolute granulocyte counts obtained daily posttransplant until death or recovery to the normal range. The shaded area represents the normal range (mean ± SD) of recovery observed in 16 dogs conditioned with 920 cGy TBI and given untreated autologous marrow (28).

marrow cells with Leu-Leu-OMe, in a concentration-dependent fashion, could reduce or eliminate in vitro CFU-GM growth. This reduction could be partially reversed with the addition of unfractionated autologous PBMC or Mφ-enriched PBMC but not with Mφ-depleted PBMC or autologous PBMC treated in vitro with Leu-Leu-OMe. Our assay employs postendotoxin-treated dog serum as a source of colony-stimulating factor. Reversal of the effects of Leu-Leu-OMe on CFU-GM growth by monocytes demonstrated the monocyte dependence of this CFU-GM assay system and suggests that PEDS does not act directly as a single factor on the granulocyte/macrophage progenitors.

Marrows were treated at two different cell concentrations in anticipation that this procedure might be useful for allogeneic marrow transplantation in large animals and possibly in man. Reduction or elimination of various cells involved either in response to alloantigen stimulation (MLC, pCTL, CTL) or NK function could be accomplished by treating PBMC or marrow cells at $20\times10^6$/ml with 1000 μM Leu-Leu-OMe. The murine models using H2-disparate P→$F_1$ marrow donor/recipient pairing showed that Leu-Leu-OMe treatment could prevent acute GVHD. The resultant chimeras developed normal cellular immune responses and showed donor and host-specific immunologic tolerance (*16*). The cell concentration treated in those studies was $2\times10^6$/ml, and the highest concentration of Leu-Leu-OMe used was 250 μM. Cell concentration directly influenced the concentration of Leu-Leu-OMe required to achieve a given effect. Thus, in the present study when the cell concentration was increased tenfold, the concentration of Leu-Leu-OMe had to be increased approximately fourfold to achieve the same inhibition of cellular immune functions. The potential problem of larger cell numbers and expanded marrow volumes needed for the transplantation of larger animals can be circumvented by increasing the concentration of Leu-Leu-OMe.

Data in the canine model suggest that Leu-Leu-OMe should be explored further as a possible substitute for other currently used methods of marrow T cell depletion. It needs to be determined whether treatment of marrow with Leu-Leu-OMe will leave behind a population of cells that facilitate engraftment and recovery of immunity while removing cytotoxic T cells that may comprise the major population of cells involved in the development of acute GVHD.

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